



Waxy gene haplotypes: Associations with pasting properties in an international rice germplasm collection[☆]

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ABSTRACT

Associations between RVA pasting properties and three single nucleotide polymorphism (SNP) sites in the *Waxy* gene intron 1, exon 6, and exon 10 were determined using rice genotypes of diverse geographic origin. A total of four SNP-haplotypes (combination of SNP alleles) were identified that explained high proportions of the variation in RVA pasting properties ($R^2 = 0.574$ – 0.704). A haplotype containing DNA sequence variation in exon 10 (exon 10 cytosine nucleotide) was exclusively found in high-apparent amylose content (AAC) genotypes with a higher RVA viscosity profile compared to the high AAC genotypes with a different haplotype. The exon 10 SNP explained variances in coolpaste and setback (coolpaste–hotpaste) to 0.642 and 0.499, respectively. Across three haplotypes, which contained exon 10 adenine nucleotide, AAC was correlated with peak, hotpaste, breakdown and setback (coolpaste–hotpaste) at $r = -0.85$, -0.75 , -0.79 , and 0.49 , respectively. Therefore, the exon 10 SNP differentiates high AAC types with a strong RVA profile and thus can be used by molecular breeding programs focused on quality improvement. Additionally, characterizing genotypes by their functional SNPs allowed us to better understand the relationship between the *Waxy* gene, its chemical product (i.e., AAC) and the functionality created by the product (i.e., pasting properties).

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1. Introduction

Rice physicochemical properties impact its functional attributes. Among the properties the apparent amylose content (AAC) and gelatinization temperature are the most used determinants for rice texture and processing properties. AAC accounts for only a small portion of milled rice starch content (0–30% by wt depending on varieties). Its synthesis is regulated by granule bound starch synthase (GBSS) which is encoded by the *Waxy* gene. Gelatinization temperature is the temperature that melts the crystalline portion of

the amylopectin structure. This starch characteristic is associated with the proportion of amylopectin short A and B1 chains, which is primarily controlled by soluble starch synthase IIa, an enzyme encoded by the *Alk* gene (Umehoto and Aoki, 2005).

Variations in end-use quality exist among rice with the same AAC or gelatinization temperature. Thus, other rice kernel constituents or their substructures also must play a role in governing aspects of rice texture and processing qualities. The super long chain of amylopectin has been associated with cooked rice texture, and RVA breakdown and setback viscosity, as well as starch granule rigidity (Bhattacharya et al., 1982; Hamaker and Griffin, 1993; Inouchi et al., 2005; Reddy et al., 1993; Sowbhagya et al., 1987). Studies have linked the content of super long chain amylopectin and starch granule rigidity to the content of GBSS (Hamaker and Griffin, 1993; Inouchi et al., 2005), which is known to be associated with several *Waxy* alleles and AAC (Hirano et al., 1998; Sano et al., 1985; Wang et al., 1995). In the U.S. some high amylose rices have superior commercial thermal processing quality that is identified using RVA coolpaste viscosity (Bergman et al., 2004) and might be associated with the sequence variation in *Waxy* gene exon 10 (Larkin and Park, 2003). Studies have also demonstrated that molecular properties of amylose, such as chain length, branching ratio, and molecular mass, affect rice pasting viscosity, rate of starch retrogradation and gel texture (Gidley and Bulpin, 1989; Mua and

Abbreviations: AAC, apparent amylose content; BD, breakdown; CPV, coolpaste viscosity; Ex6A/C SNP, adenine/cytosine single nucleotide polymorphism in *Waxy* gene exon 6; Ex10C/T SNP, cytosine/thymine single nucleotide polymorphism in *Waxy* gene exon 10; GBSS, granule bound starch synthase; HPV, hotpaste viscosity; In1G/T SNP, guanine/thymine single nucleotide polymorphism in *Waxy* gene intron 1; P_{temp} , pasting temperature; P_{time} , peak time; PV, peak viscosity; RM190 (CT_n) allele, number of cytosine–thymine dinucleotide repeat of RM190 allele; RVA, rapid visco analyzer; SB, setback; SB_HP, setback_from_hotpaste viscosity; SB_P, setback_from_peak viscosity; SNP, single nucleotide polymorphism; SSR, simple sequence repeat.

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Jackson, 1997; Shao et al., 2007). Therefore, the *Waxy* gene might impact other grain quality traits besides AAC.

Rice pasting viscosity, as determined by the rapid visco analyzer (RVA), is used by the food industry to predict its functional properties (Bason and Blakeney, 2007). It mimics the cooking process by subjecting a flour–water suspension to a heat–hold–cool–hold temperature cycle. During the heating stage, the paste viscosity rises as the temperature reaches the gelatinization temperature of amylopectin, and the granules hydrate and swell, amylose and small amylopectin leach out, and lipid complexes with amylose. The peak viscosity and the subsequent drop in viscosity during the hot holding stage have been associated with swelling and rigidity of the starch granules, which is thought to result from variations in the amylopectin, amylose, lipid and protein fractions (Fitzgerald et al., 2003; Hamaker and Griffin, 1993; Tester and Morrison, 1990). As the system cools, the viscosity rises as the soluble amylose retrogrades, forming a gel containing embedded gelatinized granules (Clark et al., 1989; Doublier et al., 1987; Miles et al., 1985).

The dynamic nature of the RVA viscosity curve makes it suitable for studying the genetic basis of grain quality. Genetic mapping studies of paste viscosity characteristics have been performed using populations developed from crosses between intermediate or high AAC lines and low AAC lines. In these studies, the largest QTL for paste viscosity measurements mapped to the *Waxy* locus on rice chromosome 6, which was also the major locus controlling AAC (Bao et al., 2000; Larkin et al., 2003; Wang et al., 2007). Bao et al. (2006a) demonstrated, in a genetic association study, a *Waxy* SNP at the first intron splice site, which discriminated low AAC rice from intermediate and high AAC rice, and was moderately associated with most of the pasting properties. When a cross between two intermediate AAC rice lines was studied, no major QTLs associated with paste viscosity characteristics were identified (Bao et al., 2002). Across these studies, the correlations of AAC to RVA pasting properties were not consistent (Bao et al., 2006b; Tan and Corke, 2002).

Gravois and Webb (1997), using a cross between two high AAC rice cultivars that have significantly different RVA pasting curves, reported that one major locus controlled these functional properties. Sequence analysis of the *Waxy* gene revealed that a single nucleotide polymorphism (SNP) in exon 10 is correlated with *Waxy* RM190, a simple sequence repeat (SSR) (Larkin and Park, 2003). The latter has been used to identify superior processing types among U.S. germplasm with high AAC rice (Bergman et al., 2001), however, the study lacked pasting viscosity data (Larkin and Park, 2003).

Using an international germplasm collection, we previously demonstrated a strong association between two functional *Waxy* SNPs, SNP at the intron 1 splice site and SNP in the exon 6, and AAC (Chen et al., 2008). These SNPs and the one in *Waxy* exon 10 might help us understand the variations in rice functional properties measured by the RVA. The genetic diversity contained within this germplasm collection makes it suitable for use in an associative genetics study designed to understand how these *Waxy* alleles contribute to the RVA viscosity curve both through the influence of AAC and other physicochemical attributes. The objectives of this study were to (1) genotype the *Waxy* exon 10 SNP of a genetically broad germplasm collection; (2) reveal the association of *Waxy* SNPs with the RVA viscosity curves; and (3) determine the portion of pasting viscosity variation explained by AAC. Haplotypes identified by this study which explain a large portion of pasting-property variation will be useful as markers in rice improvement programs.

2. Experimental

2.1. Materials

The rice accessions used in this study included 146 non-glutinous accessions of the 171 used by Chen et al. (2008) for a genetic

association study of pasting characteristics. This collection included historical and present-day U.S. cultivars as well as Asian, European, South American, and African accessions. Rough rice samples for planting were obtained from the National Small Grains Collection of the U.S. Department of Agriculture, the International Rice Research Institute Genebank and the Rice Research Unit of the USDA ARS. Field management, post-harvest handling and the genotype verification procedure were performed as described in Chen et al. (2008). All the 146 accessions were grown in 2000 and 2001 at Beaumont, Texas, and the grain collected from these two years was used for RVA viscosity and AAC determination.

2.2. Analyses of AAC and viscosity

All chemicals used were ACS-reagent grade and purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise specified. AAC was determined using the modified iodine spectrophotometric method of Pérez and Juliano (1978) with a continuous-flow analyzer (AutoAnalyzer 3, Seal Analytical, Mequon, WI, USA) (Webb, 1972). The analyses were performed with two replications and the data expressed on a 12% moisture basis. The pasting viscosity curve was measured by Rapid Visco Analyzer (Newport Scientific Model 3D, Foss Food Technology, Eden Prairie, MN, USA) using the Approved Method 61-02 of AACC International (American Association of Cereal Chemists, 2000). The parameters used to characterize the pasting curves were peak viscosity (PV), hotpaste viscosity (HPV), coolpaste viscosity (CPV), breakdown (BD), setback_from_peak viscosity (SB_P = CPV – PV), setback_from_hotpaste viscosity (SB_HP = CPV – HPV), stability (=HPV/PV) (Collado and Corke, 1997), peak time (P_{time}), and pasting temperature (P_{temp}). The pasting viscosity was determined with one replication per year and the two-year data was treated as replicates for the statistical analysis.

2.3. Genotyping methods

The genomic DNA of each accession was extracted using a CTAB extraction procedure of Fjellstrom et al. (2004) from leaf material collected from the field plots grown in 2000.

The sequence variations in exon 1 of RM190 ((CT)_n alleles), G/T polymorphism in intron 1 of the *Waxy* gene (In1G/T SNP), and sequence variation in exon 6 (Ex6A/C SNP) were genotyped using RM190 markers, restriction enzyme digestion of the PCR amplified fragment surrounding the intron 1 splice site, and the dideoxy fingerprinting method of PCR fragments surrounding the intron 5, exon 6 and intron 6 regions, respectively, as described in Chen et al. (2008).

The sequence variation in exon 10 (Ex10C/T SNP) was genotyped using a dideoxy fingerprinting method. Rice genomic DNA was the template for PCR amplification of *Waxy* gene sequences. The forward primer was 21633-2Fb (5'-TTTGAAAAAGAAATTATCATCTGTCAC-3'), and the reverse primer was 21633-2R (5'-CTGCATGAGCTCCGG-GATG-3'). The PCR reaction was as follows: DNA denaturation at 94 °C for 5 min; followed by 30 amplification cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min; and then extension at 72 °C for 7 min. The PCR reactions were performed in a 20-μL volume containing 0.2 mM dNTPs, 2.5 mM MgCl₂, 0.1 μM of forward and reverse primers, approximately 5 ng genomic DNA, and 0.8 U Tfl polymerase. The amplified PCR product was then diluted 1:5 with water, and 1 μL used as a DNA template for a dideoxy-CTP termination reaction with an IRD700-labeled forward primer (5'-TCAGGCAATC-GAGGCGAAG-3'). Each termination reaction contained 0.65 pmol IRD-labeled primer, 2.7 U of Thermo Sequenase DNA Polymerase, 3 μL ddC Termination Mix (Thermo Sequenase Cycle Sequencing Kit, USB Corp.), and 0.1× reaction buffer (Thermo Sequenase Cycle Sequencing Kit, USB Corp.). The thermocycle of the termination

Table 1
Alleles and numbers of accessions associated with *Waxy* SNP-haplotypes

Waxy SNP-haplotype ^a	No. of accessions	Allele of Waxy SNPs			No. of accessions of Waxy RM190 alleles ^b									
		In1	Ex6	Ex10	CT ₈	CT ₁₀	CT ₁₁	CT ₁₄	CT ₁₆	CT ₁₇	CT ₁₈	CT ₁₉	CT ₂₀	
Waxy-L	52	T	A	C						10	37	5		
Waxy-I	53	G	C	C				5	5	7	4	4	28	
Waxy-H	20	G	A	C	6	9	3						2	
Waxy-HH	21	G	A	T			21							

^a *Waxy* SNP-haplotype: combination of alleles of *Waxy* In1, Ex6 and Ex10 SNPs.

^b *Waxy* RM190 alleles: number of cytosine–thymine dinucleotide repeat (CT_n).

reaction was denaturation at 95 °C for 2 min, 30 amplification cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s, and extension at 72 °C for 5 min. After the termination reaction, 3 µl IR² stop solution (Li-Cor, Inc) was added. The mixture was denatured for 3 min at 92 °C and electrophoresed on a 0.5× MDE gel (CAMBREX Bio Science Rockland Inc., Rockland, ME, USA). The electrophoresis and detection of the labeled bands were performed on a Li-Cor IR² DNA Sequencer (Li-Cor, Inc.). The run setting of the Li-Cor system was 2000 V, current of 25 A, power of 12 W and run time of 16 h. The running buffer was 0.6× TBE.

2.4. Statistics

All statistical analyses were performed using SAS version 8.2 (SAS Institute Inc., Cary, NC). The PROC GLM was used for analysis of variance determination, and mean separation analysis was carried out using the Student–Newman–Keuls test. The PROC CORR was used to perform Pearson's product moment correlation analysis.

3. Results and discussion

3.1. *Waxy* SNP-haplotypes and the origin of rice accessions

Three SNP sites in the *Waxy* gene, In1G/T, Ex6A/C, and Ex10C/T, were genotyped. Together, a total of four haplotypes or alleles were identified in the germplasm studied and are designated as *Waxy*-L, *Waxy*-I, *Waxy*-H, and *Waxy*-HH (Table 1). The geographic origins of all the rice accessions are presented in Table 2. Approximately half of the accessions containing *Waxy*-L or *Waxy*-I were from North America, while the others were from other global regions. The *Waxy*-H and *Waxy*-HH were from different parts of Asia. All four haplotypes were found in most rice producing regions of the world.

3.2. Association of *Waxy* SNPs with viscosity curve properties and AAC

The *Waxy* SNP-haplotypes significantly ($P < 0.001$) explain a large portion of the variance of the pasting properties ($R^2 = 0.574$ – 0.704) (Table 3). These properties include the specific absolute viscosity of PV, HPV, CPV, and the derived properties of BD, SB_P, SB_HP, and stability (Table 3). The SNP-haplotypes explain some of the P_{time} variation ($R^2 = 0.266$, $P < 0.001$), but not the P_{temp} . The P_{temp} gives an indication of the gelatinization temperature of

starch. Soluble starch synthase IIa controls most of the variation in gelatinization temperature, thus it is not surprising that functional sequence changes in the *Waxy* gene did not explain much of the variation in P_{temp} (Umemoto and Aoki, 2005).

Previously, we identified three *Waxy* SNP-haplotypes that were strongly associated with AAC (Chen et al., 2008). The SNPs evaluated in that study were In1G/T and Ex6A/C of the *Waxy* gene. The additional SNP included in this study was a nucleotide substitution of cytosine to thymine in exon 10 (Ex10C/T). The rice accessions having In1T–Ex6A and In1G–Ex6C haplotypes in Chen et al. (2008) all contained the Ex10C allele, and were named as SNP-haplotypes of *Waxy*-L and *Waxy*-I, respectively, in this report (Table 1). The SNP Ex10T allele was found only in high AAC rice (In1G–Ex6A haplotype in Chen et al. (2008)), and contributed to one additional haplotype, *Waxy*-HH, among the non-glutinous high AAC accessions (Table 1). The four *Waxy* SNP-haplotypes explained 86.6% of the total variation in AAC, which is about the same amount of variance explained by the combination of the *Waxy* SNPs of In1 and Ex6 ($R^2 = 0.864$) of this data set (Table 3). Therefore, the Ex10 polymorphism has little effect on AAC.

The variances in pasting properties explained by each SNP or their combination are listed in Table 3. The explained variances in BD, SB_P, SB_HP and stability increased from the In1 SNP (distinguishes low from intermediate and high AAC types) to the In1–Ex6, which coincided with the increase of variance in AAC, thus suggesting the strong correlations of these pasting-property parameters with the AAC (see Section 3.3). Comparing the variances in HPV and CPV explained by the In1, the In1–Ex6 and the SNP-haplotypes (In1–Ex6–Ex10), it is evident that the inclusion of the Ex10 SNP in the SNP-haplotypes significantly increased the amount of variance explained for these two traits, which implies that the Ex10 SNP resolved the confounding effect residing in the high AAC types (Table 3). The Ex10 SNP provides an additive effect to the SNP-haplotypes by explaining the variances in SB_P, SB_HP and stability, but provides no additive effect to the SNP-haplotypes associated with BD.

Mean separation of AAC among haplotypes (Table 3) demonstrated that *Waxy*-L contained accessions with low AAC (mean AAC = 14.8%), *Waxy*-I consisted of those with intermediate AAC (mean AAC = 21.0%), and both *Waxy*-H and *Waxy*-HH had high AAC types. The *Waxy*-H had slightly higher AAC (mean AAC = 24.8%) than *Waxy*-HH (mean AAC = 24.1%) ($\alpha = 0.05$). The combination of the In1 and Ex6-SNPs is able to classify rice into the following AAC classes: low: 5–18%, intermediate: 19–23% and high: >23%.

Table 2
Origin of rice accessions by *Waxy* SNP-haplotypes

<i>Waxy</i> SNP-haplotype ^a	Africa	Australia	Europe	Central America	Northern America	Southern America	Eastern Asia	Southeastern Asia	Southern Asia	Southwestern Asia	Total
<i>Waxy</i> -L	2	2	8	1	22	2	10	4	1	0	52
<i>Waxy</i> -I	6	2	1	2	25	2	2	7	5	1	53
<i>Waxy</i> -H	0	0	1	0	2	0	4	2	11	0	20
<i>Waxy</i> -HH	1	0	0	2	2	3	6	3	3	1	21
Total	9	4	10	5	51	7	22	16	20	2	146

^a *Waxy* SNP-haplotype: combination of alleles of *Waxy* In1, Ex6 and Ex10 SNPs.

Table 3Trait variances explained by *Waxy* RM190, SNPs, combination of SNPs, SNP-haplotypes and the mean trait values of *Waxy* SNP-haplotypes

Trait	Variance (R^2)					SNP-hap	Mean ^a	Trait	Variance (R^2)					SNP-hap	Mean ^a
	RM190	ln1	ln1–Ex6	Ex10	SNP-hap				RM190	ln1	ln1–Ex6	Ex10	SNP-hap		
AAC (%)	0.740***	0.762***	0.864***	0.174***	0.866***			SB_P (RVU)	0.544***	0.448***	0.555***	0.353***	0.625***		
						Waxy-L	14.8 ^D							Waxy-L	15 ^D
						Waxy-I	21.0 ^C							Waxy-I	78 ^C
						Waxy-H	24.8 ^A							Waxy-H	97 ^B
						Waxy-HH	24.1 ^B							Waxy-HH	157 ^A
PV (RVU)	0.428***	0.407***	0.410***	0.011	0.604***			SB_HP (RVU)	0.540***	0.219***	0.303***	0.499***	0.578***		
						Waxy-L	305 ^A							Waxy-L	135 ^D
						Waxy-I	236 ^C							Waxy-I	155 ^B
						Waxy-H	183 ^D							Waxy-H	146 ^C
						Waxy-HH	273 ^B							Waxy-HH	205 ^A
HPV (RVU)	0.525***	0.061***	0.124***	0.398***	0.674***			Stability	0.507***	0.321***	0.502***	0.401***	0.582***		
						Waxy-L	185 ^B							Waxy-L	0.61 ^D
						Waxy-I	159 ^C							Waxy-I	0.68 ^C
						Waxy-H	133 ^D							Waxy-H	0.73 ^B
						Waxy-HH	225 ^A							Waxy-HH	0.83 ^A
CPV (RVU)	0.612***	0.013	0.118***	0.642***	0.704***			P_{time} (min)	0.210***	0.018*	0.054***	0.173***	0.266***		
						Waxy-L	320 ^B							Waxy-L	5.84 ^B
						Waxy-I	314 ^B							Waxy-I	5.76 ^C
						Waxy-H	280 ^C							Waxy-H	5.69 ^D
						Waxy-HH	430 ^A							Waxy-HH	5.97 ^A
BD (RVU)	0.456***	0.487***	0.574***	0.153***	0.574***			P_{temp} (°C)	0.036	0.016*	0.019	0.004	0.020		
						Waxy-L	120 ^A							Waxy-L	84.0 ^A
						Waxy-I	77 ^B							Waxy-I	84.6 ^A
						Waxy-H	50 ^C							Waxy-H	85.1 ^A
						Waxy-HH	48 ^C							Waxy-HH	84.9 ^A

AAC, apparent amylose content; PV, peak viscosity; HPV, hotpaste viscosity; CPV, coolpaste viscosity; BD, breakdown; SB_P, setback from peak viscosity; SB_HP, setback from hotpaste viscosity; P_{time} , peak time; P_{temp} , pasting temperature.

*, ** and *** = Significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

^a Mean separation analysis of traits is determined by the Student–Newman–Keuls test. Means with different superscript letters indicate significance at $\alpha = 0.05$.

The mean separation analysis (Table 3) of the pasting parameters among SNP-haplotypes shows that most of the viscosity parameters except BD were significantly different between *Waxy*-H and *Waxy*-HH rice. The *Waxy*-HH had the highest viscosities in HPV, CPV, SB_P, and SB_HP, stability, and P_{time} , and second highest in PV, less than that of *Waxy*-L. Both *Waxy*-H and *Waxy*-HH had low BD. BD is a measure of susceptibility to shear thinning and starch granule rigidity, but has been reported to fail in prediction of certain quality types of rice among

high AAC types (Bhattacharya and Sowbhagya, 1978). The higher viscosity of *Waxy*-HH rice might be in part due to the rigidity of the starch granule (Rani and Bhattacharya, 1995). This hypothesis was supported by the higher P_{time} , the time that shear and swelling was balanced during heating stage, of *Waxy*-HH than the other haplotypes (Table 2).

Differences in functional property among high AAC rice have been reported. The cooked rice texture differed among high AAC rice, and has been associated with the content of hot-water-insoluble

Table 4

Pearson's correlation coefficients between AAC and RVA viscosity curve parameters

Traits ^a	Accessions ^b	AAC	PV	HPV	CPV	BD	SB_P	SB_HP	Stability	P_{time}
PV	All	–0.70***								
	<i>Waxy</i> -L, -I, -H	–0.85***								
HPV	All	–0.23***	0.72***							
	<i>Waxy</i> -L, -I, -H	–0.75***	0.87***							
CPV	All	0.18**	0.35***	0.85***						
	<i>Waxy</i> -L, -I, -H	–0.34***	0.47***	0.75***						
BD	All	–0.80***	0.80***	0.17**	–0.23***					
	<i>Waxy</i> -L, -I, -H	–0.79***	0.94***	0.64***	0.19**					
SB_P	All	0.78***	–0.59***	0.08	0.55***	–0.92***				
	<i>Waxy</i> -L, -I, -H	0.76***	–0.86***	–0.55***	0.05	–0.95***				
SB_HP	All	0.57***	–0.18**	0.39***	0.82***	–0.58***	0.86***			
	<i>Waxy</i> -L, -I, -H	0.49***	–0.47***	–0.22***	0.48***	–0.58***	0.81***			
Stability	All	0.67***	–0.49***	0.23***	0.54***	–0.90***	0.90***	0.68***		
	<i>Waxy</i> -L, -I, -H	0.63***	–0.75***	–0.35***	0.05	–0.92***	0.87***	0.54***		
P_{time}	All	–0.06	0.21***	0.60***	0.54***	–0.22***	0.28***	0.28***	0.48***	
	<i>Waxy</i> -L, -I, -H	–0.30***	0.22***	0.51***	0.47***	–0.02	0.02	0.01	0.27***	
P_{temp}	All	0.16**	–0.44***	–0.10	0.12*	–0.54***	0.50***	0.32***	0.51***	0.30***
	<i>Waxy</i> -L, -I, -H	0.16**	–0.45***	–0.21***	0.10	–0.54***	0.56***	0.44***	0.59***	0.27***

*, ** and *** = Significance at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively.

^a Trait abbreviations are the same as those in Table 3.

^b All: includes accessions of all haplotypes (*Waxy*-L, *Waxy*-I, *Waxy*-H and *Waxy*-HH); *Waxy*-L, -I, -H: includes accessions of *Waxy*-L, *Waxy*-I, *Waxy*-H haplotypes.

amylose, long B chains of amylopectin, rigidity of the starch granule, and the amount of GBSS (Bhattacharya et al., 1982; Rani and Bhattacharya, 1995; Reddy et al., 1993; Sowbhagya et al., 1987). We have demonstrated that the RVA profiles differ between the two high AAC types and are associated with the *Waxy* gene Ex10C SNP.

3.3. Correlations of AAC and RVA viscosity curve parameters

Two sets of coefficients for the correlation analyses between AAC and all viscosity curve parameters are presented in Table 4. The first set includes all rice accessions. The second set comprises all rice that has an Ex10C allele, which includes *Waxy*-L, *Waxy*-I and *Waxy*-H accessions. The reason for having the second set of data is to see the effect of AAC on pasting properties without adding the confounding factors of rice with the special processing quality of

Waxy-HH. Scatter plots of AAC versus pasting parameters (i.e., PV, HPV, CPV, BD, SB_P, SB_HP and stability) by *Waxy* gene haplotype are presented in Fig. 1.

AAC correlated with most of the pasting parameters. The PV of *Waxy*-L, *Waxy*-I and *Waxy*-H accessions was negatively correlated with AAC, $r = -0.85^{***}$ (Table 4 and Fig. 1a). The HPV of *Waxy*-L, *Waxy*-I and *Waxy*-H accessions was negatively associated with AAC ($r = -0.75^{***}$) (Fig. 1b and Table 4). In contrast to this, the high AAC *Waxy*-HH had the highest HPV. The BD and SB_P correlated well with AAC ($r = -0.80^{***}$ and 0.78^{***}) across all the rice accessions (Fig. 1d and e; Table 4). The SB_HP and stability had higher correlations with AAC when all the rice accessions were included in the analysis; the correlation coefficients were $r = 0.57$ and $r = 0.67$, respectively. The scatter plots (Fig. 1) and mean separation analysis (Table 3) indicate that *Waxy*-H, even though it has the highest AAC,

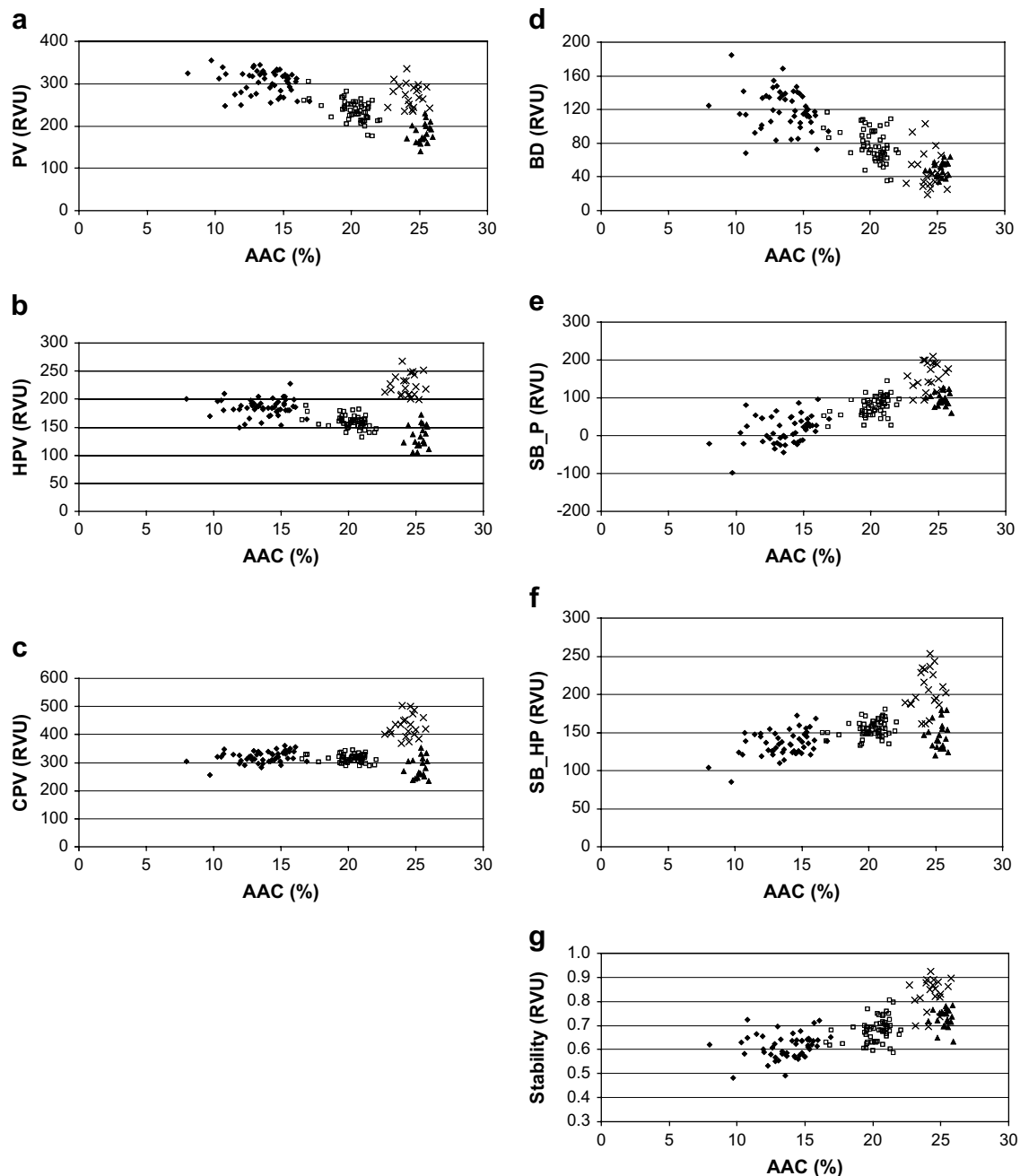


Fig. 1. Scatter plots of the viscosity curve parameters against apparent amylose content (%) (AAC) of the non-glutinous rice accessions. Each data point was the average of the two years' values. Symbols are: \blacklozenge , *Waxy*-L accessions; \square , *Waxy*-I accessions; \blacktriangle , *Waxy*-H accessions; \times , *Waxy*-HH accessions.

had lower SB_HP than it would be hypothesized to have based solely on its AAC. The CPV did not correlate well with AAC across all rice accessions ($r = 0.18$) nor in the *Waxy*-L, *Waxy*-I and *Waxy*-H rice types ($r = -0.34$) (Fig. 1c and Table 4), however, it strongly associated with the Ex10 polymorphism. Viscosity parameters of PV, BD and SB_P have high correlation coefficients with AAC and also correlate well with each other (Table 4). The SB_HP and CPV had high correlation with each other across all accessions; while each of them had moderate and low correlation with AAC, respectively, suggesting the close functional link between them with minimal impact from AAC.

The negative correlation of PV and HPV with AAC in rice with the Ex10C allele might be due to amylose that resists starch granule swelling (Tester and Morrison, 1990). It is possible that the physicochemical changes caused by the Ex10 polymorphism in *Waxy*-HH increase both PV and HPV, although more so for HPV. The HPV is affected by shear thinning, and the realignment of polymers in the continuous phase of the paste (Bergman et al., 2004). Factor analysis grouped PV into two factors suggesting that there are more variables contributing to PV than to HPV (Tan and Corke, 2002). SB_HP is a measure of the short-term retrogradation of starch paste. It was positively correlated with AAC. The *Waxy*-HH and

Table 5
Mean values of AAC and RVA viscosity curve parameters of U.S. and non-U.S. rice

Traits ^a	Waxy SNP-haplotype	U.S.				Non-U.S.			
		<i>n</i>	Mean \pm SD ^b	Min	Max	<i>n</i>	Mean \pm SD ^b	Min	Max
AAC (%)	All	51	17.8 \pm 5.3	0.0	25.4	95	18.2 \pm 7.5	0.0	26.0
	<i>Waxy</i> -L	22	14.4 \pm 2.2 ^A	10.3	19.2	30	15.0 \pm 2.1 ^A	8.0	19.7
	<i>Waxy</i> -I	25	21.1 \pm 1.3 ^A	16.6	23.0	28	20.9 \pm 1.3 ^A	16.9	23.1
	<i>Waxy</i> -H	2	24.9 \pm 0.4	24.5	25.4	18	24.8 \pm 0.8	22.7	26.0
	<i>Waxy</i> -HH	2	24.7 \pm 0.6	23.9	25.3	19	24.0 \pm 0.8	21.9	25.8
PV (RVU)	All	51	269 \pm 50	160	369	95	254 \pm 56	133	402
	<i>Waxy</i> -L	22	310 \pm 37 ^A	233	369	30	302 \pm 42 ^A	207	391
	<i>Waxy</i> -I	25	238 \pm 29 ^A	187	307	28	235 \pm 33 ^A	164	325
	<i>Waxy</i> -H	2	186 \pm 19	160	203	18	183 \pm 29	135	245
	<i>Waxy</i> -HH	2	281 \pm 26	260	318	19	272 \pm 32	223	353
HPV (RVU)	All	51	174 \pm 24	116	256	95	172 \pm 38	62	284
	<i>Waxy</i> -L	22	188 \pm 14 ^A	150	216	30	183 \pm 21 ^A	138	240
	<i>Waxy</i> -I	25	161 \pm 16 ^A	127	198	28	158 \pm 16 ^A	121	202
	<i>Waxy</i> -H	2	143 \pm 11	132	155	18	132 \pm 24	93	190
	<i>Waxy</i> -HH	2	221 \pm 28	196	256	19	225 \pm 21	194	284
CPV (RVU)	All	51	320 \pm 39	146	526	95	316 \pm 75	78	533
	<i>Waxy</i> -L	22	324 \pm 17 ^A	292	366	30	318 \pm 25 ^A	254	372
	<i>Waxy</i> -I	25	319 \pm 17 ^A	276	352	28	309 \pm 19 ^B	260	366
	<i>Waxy</i> -H	2	283 \pm 25	255	311	18	279 \pm 44	217	383
	<i>Waxy</i> -HH	2	436 \pm 67	380	526	19	429 \pm 41	343	533
BD (RVU)	All	51	94 \pm 34	28	178	95	81 \pm 38	12	216
	<i>Waxy</i> -L	22	122 \pm 29 ^A	64	178	30	119 \pm 32 ^A	56	216
	<i>Waxy</i> -I	25	77 \pm 19 ^A	44	127	28	77 \pm 24 ^A	31	132
	<i>Waxy</i> -H	2	43 \pm 10	28	49	18	51 \pm 12	18	80
	<i>Waxy</i> -HH	2	60 \pm 9	46	67	19	47 \pm 25	12	116
SB_P (RVU)	All	51	52 \pm 56	-92	208	95	62 \pm 73	-137	219
	<i>Waxy</i> -L	22	14 \pm 45 ^A	-65	98	30	16 \pm 49 ^A	-137	109
	<i>Waxy</i> -I	25	82 \pm 26 ^A	6	118	28	74 \pm 33 ^A	4	155
	<i>Waxy</i> -H	2	97 \pm 11	86	113	18	97 \pm 23	47	147
	<i>Waxy</i> -HH	2	155 \pm 42	120	208	19	157 \pm 38	71	219
SB_HP (RVU)	All	51	146 \pm 31	30	271	95	143 \pm 49	16	263
	<i>Waxy</i> -L	22	136 \pm 17 ^A	103	173	30	134 \pm 20 ^A	80	179
	<i>Waxy</i> -I	25	158 \pm 10 ^A	133	180	28	152 \pm 14 ^B	127	190
	<i>Waxy</i> -H	2	140 \pm 15	123	160	18	147 \pm 23	115	207
	<i>Waxy</i> -HH	2	215 \pm 40	184	271	19	204 \pm 29	138	263
Stability	All	51	0.66 \pm 0.07	0.52	0.83	95	0.69 \pm 0.10	0.45	0.95
	<i>Waxy</i> -L	22	0.61 \pm 0.05 ^A	0.52	0.74	30	0.61 \pm 0.06 ^A	0.45	0.75
	<i>Waxy</i> -I	25	0.68 \pm 0.05 ^A	0.56	0.80	28	0.68 \pm 0.07 ^A	0.57	0.83
	<i>Waxy</i> -H	2	0.77 \pm 0.04	0.73	0.83	18	0.72 \pm 0.06	0.61	0.87
	<i>Waxy</i> -HH	2	0.79 \pm 0.04	0.75	0.83	19	0.83 \pm 0.07	0.65	0.95
<i>P</i> _{time} (min)	All	51	5.76 \pm 0.29	3.87	6.07	95	5.64 \pm 0.52	3.60	6.20
	<i>Waxy</i> -L	22	5.86 \pm 0.11 ^A	5.60	6.07	30	5.82 \pm 0.17 ^A	5.33	6.20
	<i>Waxy</i> -I	25	5.75 \pm 0.12 ^A	5.53	6.00	28	5.76 \pm 0.15 ^A	5.40	6.07
	<i>Waxy</i> -H	2	5.83 \pm 0.09	5.73	5.93	18	5.67 \pm 0.12	5.47	5.93
	<i>Waxy</i> -HH	2	5.87 \pm 0.05	5.80	5.93	19	5.98 \pm 0.15	5.53	6.20
<i>P</i> _{temp} (°C)	All	51	84.5 \pm 3.2	71.2	88.9	95	83.2 \pm 4.6	67.4	91.0
	<i>Waxy</i> -L	22	84.3 \pm 3.2 ^A	77.4	88.9	30	83.8 \pm 3.0 ^A	77.5	88.9
	<i>Waxy</i> -I	25	85.2 \pm 2.3 ^A	80.7	88.9	28	84.0 \pm 3.5 ^B	75.3	91.0
	<i>Waxy</i> -H	2	84.7 \pm 0.6	83.9	85.4	18	85.1 \pm 2.5	79.3	89.4
	<i>Waxy</i> -HH	2	85.2 \pm 1.5	83.1	86.2	19	84.9 \pm 2.8	76.1	88.9

Different letters indicate there is a significance difference ($\alpha = 0.05$) between U.S. and non-U.S. of each SNP-haplotype.

^a Trait abbreviations are the same as those in Table 3.

^b Mean separation analysis of traits is determined by the Student–Newman–Keuls test. The analyses were done for *Waxy*-L and *Waxy*-I accessions only.

Waxy-I haplotypes had significantly higher SB_HP than those accessions in Waxy-H suggesting that other factor(s) in addition to AAC might be contributing to the SB_HP property. This process of amylose forming a crosslinked network (gelation) is both concentration and chain-length dependent (Gidley and Bulpin, 1989). It is known that the M_w of rice amylose vary among rice cultivars with different AAC (Park et al., 2007). The SB_HP has also been correlated to the content of the very long chains of amylopectin (Inouchi et al., 2005).

3.4. Associations of Waxy SNP-haplotypes and Waxy RM190 SSR alleles

The association of the Waxy SNP-haplotypes with the Waxy RM190 marker is presented in Table 1. The majority of the rice containing Waxy-L has the RM190 CT₁₇ and CT₁₈ alleles, while the others have CT₁₉. The majority of accessions in the Waxy-I group has the CT₂₀ allele, but others have the CT repeats of 14, 16, 17, 18 and 19. The Waxy-H accessions contain the shorter CT repeats of 8, 10 and 11. All Waxy-HH rice accessions have a CT₁₁ allele.

Rice varieties with a strong RVA profile are sometimes referred to in the U.S. as superior processing types. The U.S. breeding lines and cultivars with this quality were reported to contain the Waxy RM190-(CT₁₁) allele (Ayres et al., 1997; Bergman et al., 2001). This association was not as strong when the rice accessions were expanded to an international rice collection. All accessions in the Waxy-HH have an RM190-(CT₁₁) allele but the reverse is not true. Mean separation analysis (Table 3) showed that Waxy-HH accessions had a stronger RVA viscosity curve than the Waxy-H accessions. The two-year-averages of CPV of the three Waxy-H rice accessions containing an RM190-(CT₁₁) allele were 245, 333, and 351 RVU. These values are within the CPV range of Waxy-H rice (average of the two years), which was 234–351 RVU. The CPV range (average of the two years) of Waxy-HH rice was 370–502 RVU.

The Waxy RM190 is a DNA microsatellite marker located in the non-coding region of Waxy gene exon 1. This marker strongly associated with AAC across U.S. cultivars, breeding lines and germplasm (Ayres et al., 1997; Bergman et al., 2001). This association was not as strong when the rice accessions were expanded to an international rice collection (Bergman et al., 2000); while the SNP-haplotypes (combination of In1 and Ex6-SNPs) explained more of the variation in AAC across environments using this same germplasm collection (Chen et al., 2008). The strong association of the functional SNPs in Waxy gene In1 and Ex6 with quality traits across rice of diverse origins was also demonstrated here for the Ex10 SNP. Thus the Waxy SNP-haplotypes, as compared to Waxy RM190, would have superior utility as a molecular marker for making end-use quality selections in breeding programs, especially those that utilize a wide germplasm base.

3.5. Means and ranges of AAC and pasting properties of U.S. and non-U.S. rice and molecular markers for pasting properties

AAC and pasting properties of all the rice accessions are shown in Table 5 divided by their sub-groups: U.S. and non-U.S. origins, and also further divided by the haplotypes within each sub-group. U.S. rice had a narrower range in trait values compared to non-U.S. accessions. Most of the traits of U.S. rice fall within the ranges of those of non-U.S. rice. Comparing the trait values of each Waxy SNP-haplotype U.S. rice had narrower ranges compared to non-U.S. rice. Analysis of variance demonstrated that the proportion of variance explained by the haplotypes was 0.820, 0.586, 0.555, 0.581, 0.538, 0.551, 0.558, 0.491, and 0.187 for AAC, PV, HPV, CPV, BD, SB_P, SB_HP, stability and P_{time} , respectively, in U.S. rice, while the proportion of variance explained by the haplotypes in non-U.S. rice was 0.882, 0.606, 0.704, 0.727, 0.571, 0.641, 0.587, 0.593 and 0.315,

respectively. The mean separation analyses of AAC and pasting-property parameters of Waxy-L and Waxy-I showed that most of the parameters had no significant differences ($\alpha = 0.05$) between U.S. and non-U.S. rice (Table 5).

Based on a population structure study, U.S. rice germplasm structure was established before 1930 and remains essentially intact today. It is clustered into three groups: temperate *japonica* with short to medium grains, tropical *japonica* with medium grains, and tropical *japonica* with long grains (Lu et al., 2005). The mean trait values of this relatively narrow genetic pool of rice were not significantly different from the non-U.S. rice suggesting the functional role and wide utility of these Waxy SNPs as molecular markers for rice grain quality trait selection.

4. Conclusion

We have demonstrated that sequence changes in the rice Waxy gene explain a large proportion of the variances in RVA viscosity properties and in AAC within a genetically diverse set of rice accessions. Single nucleotide polymorphic sites within the Waxy gene that associated with AAC were distinct from the SNP controlling the superior processing property of rice in the high AAC class. Characterizing rice accessions by their Waxy SNP-haplotypes provided a clear picture of how AAC contributes to the pasting properties without being obscured by Waxy-HH type. As we reported earlier, the combination of Waxy SNPs of In1 and Ex6 offers breeding programs a good molecular marker for selecting for AAC. We are now able to add an additional marker, the Ex10 SNP, for selection of rice with a strong pasting profile.

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